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Immunology

A strategy for organ allografts without using immunosuppressants or irradiation

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ABSTRACT **A strategy to achieve regular and long lasting organ and tissue allografts without using immunosuppressants and/or irradiation has been established for mice. One hundred percent of skin allografts can be induced to survive >350 days after transplantation if spleen cells from the same donors are first injected into the portal vein of the recipients. The mechanisms underlying this long-term tolerance induction can be described as follows: (*i*) donor T cells from the spleen of the donor facilitate the acceptance of the allogeneic engraftment, (*ii*) donor-specific anergy is induced in the cytotoxic T-lymphocytes of the recipients, (*iii*) T helper type 2 cells become the dominant T cells in the recipients that are accepting the skin transplants, and (*iv*) a lasting chimerism (microchimerism) is established in these recipients. This strategy, perhaps with minor modifications, might permit one also to overcome major barriers to organ allografting in humans. If this were the case, it could represent production of long lasting immunologic tolerance without need for irradiation or cytotoxic chemo-preparative regimen and as such could greatly facilitate allotransplantation free of episodes of chronic or acute rejection or toxic and damaging preparatory regimens.**

Various attempts have been made to induce persistent immunologic tolerance across major histocompatibility complex (MHC) barriers. Immunologic tolerance is generally induced by three mechanisms: clonal deletion, cellular suppression, and clonal anergy. Clonal deletion, which contributes a major component of self-tolerance, is induced by reconstituting lethally irradiated recipients with donor hematolymphoid cells (1); we reported earlier (2–4) that successful organ allografts can be achieved by carrying out bone marrow transplantation in conjunction with organ allografts. Improvements on this strategy have been recently made using mAbs against T cells to reduce radiation doses needed (5) and/or to reduce bodily areas exposed to irradiation (6, 7). On the other hand, it recently has been noted that potent and persistent immunologic tolerance is induced using clonal anergy and suppression mechanisms (8, 9). In these reports, anergy to cardiac allografts was induced, for example, by CD28-B7 blockade or the expression of "protective genes"; suppression of graft-reactive T helper type 1 (Th1) cells has been maintained by the expansion of T helper type 2 (Th2) cells.

Portal venous (p.v.) administration of foreign cells has been reported to induce donor-specific tolerance across major (10–15), minor (16), and xeno (17) histocompatibility complex (HC) barriers. We recently have analyzed the mechanism by which the donor-specific tolerance is induced after p.v. injection and have found this form of tolerance to be attributable to induction of clonal anergy in the $CD8^+$ T cells of the recipients (18). In the present study, using initially a p.v. injection of spleen cells (SPLCs) or bone marrow cells (BMCs) plus i.v. injection of hematopoietic bone marrow cells followed by application of skin allografts, we present a protocol for the induction of potent and durable immunologic tolerance across MHC barriers.

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6, H-2^b), BALB/c (H-2^d), C3H/He (H-2^k), and (BALB/c x DBA/2)F1 (CDF1) (H-2^d) mice were obtained from Japan SLC (Hamamatsu, Japan). These mice were used at 8–10 weeks of age.

Preparation of Allogeneic Cells and Injection Via a p.v. Route. BMCs were collected from the femoral and tibial bones of donor mice. SPLCs were suspended as a single cell suspension that was used after lysis of erythrocytes with Tris-ammonium chloride buffer (pH 7.4). The T cell-enriched population of SPLCs was obtained by incubating in a nylon–wool fiber (Wako Pure Chemical, Osaka) column for 90 min at 37°C: 1 g of the nylon–wool fiber was prepared for 2×10^8 SPLCs. After the incubation, 85–90% of the cells eluted from the nylon–wool fiber column were CD3-positive. The T cell-depleted population of spleen cells was obtained by using mAbs against CD4 (clone GK1.5, rat IgG2b, American Type Culture Collection, ATCC), CD8 (clone 53–6.72 rat IgG2b, ATCC), and sheep-anti-rat IgG immunomagnetic beads, IgG (Dynabeads M450; Dynal Oslo, Norway). The resultant cell population contained $< 0.5\%$ of CD4 or CD8-positive cells. The cells were injected via the p.v. as described (19). In brief, donor cells (3×10^7) in 0.2–0.3 ml of RPMI 1640 medium were injected through the superior mesenteric vein using a 27-gauge needle. After the injection, hemostasis was secured by gentle pressure with a cotton–wool swab.

Injection of Immunosuppressants. Cyclosporin A (CsA; Sandimmun, Novartis, Basel, Switzerland; 10 mg/kg) or FK506 (Fujisawa Pharmaceutical, Osaka; 1 mg/kg) were injected i.p. into recipient mice on day 2 and/or day 5 after the p.v. injection of the allogeneic SPLCs or BMCs.

Skin Grafting. The skin grafting was performed 7 days after the p.v. injection according to the method of Mayumi and Good (20) with minor modifications. Full-thickness skin grafts

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Abbreviations: CTL, cytotoxic T lymphocyte; HC, histocompatibility complex; MHC, major HC; SPLC, spleen cell; BMC, bone marrow cell; p.v. portal venous; i.v. peripheral intravenous; Th1, T helper type 1; Th2, T helper type 2; HSC, hematopoietic stem cell; CsA, cyclosporin A.

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(1 cm × 1.5 cm) were harvested from the dorsal wall of donor from which hair had been completely removed by plucking and using depilatory. Skin grafts were sutured to the graft beds on the right thoracic wall by using 6–0 nylon and covered with Vaseline gauze and protective tape. The first inspection of the skin graft was carried out 14 days after grafting, and thereafter, inspections of the skin graft were made at least twice a week. Because the graft rejection started with loss of hair and culminated in necrosis of the entire graft skin, the graft was considered to have been rejected when no normal epithelium could be found in the graft beds. In otherwise untreated C57BL/6 mice, BALB/c skin grafts were regularly completely rejected within 14 days after transplantation.

Assays for Mixed Leukocyte Responses (MLR) and the Generation of Cytotoxic T Lymphocytes (CTLs). In MLRs, the isolated $CD4^+$ or $CD8^+$ cells (as responders) were incubated for 48 hr with irradiated (15 Gy) lipopolysaccharide-blasts (1×10^5 as stimulators). The $CD8^+$ or $CD4^+$ cells were isolated by using the magnetic cell separation system (magnetic cell sorter II, Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany) and anti-mouse CD8/CD4 mAb-conjugated ferritdextran (anti-mouse CD8/CD4 microbeads, Miltenyi Biotec) as described by Miltenyi *et al.* (21). The responses were evaluated by measuring the uptake of [^{3}H]thymidine. The generation of CTLs was evaluated by chromium (^{51}Cr) release from labeled target cells, P815 (H-2^d) or BW5147 (H-2^k) by CTLs generated in MLRs in which 3×10^6 $CD8^+$ cells isolated from the recipients were incubated for 5 days with 3×10^6-irradiated lipopolysaccharide-blasts prepared from BALB/c (H-2^d) or C3H (H-2^k) mice. The CTL activity is expressed as "percent-specific lysis," as described (1).

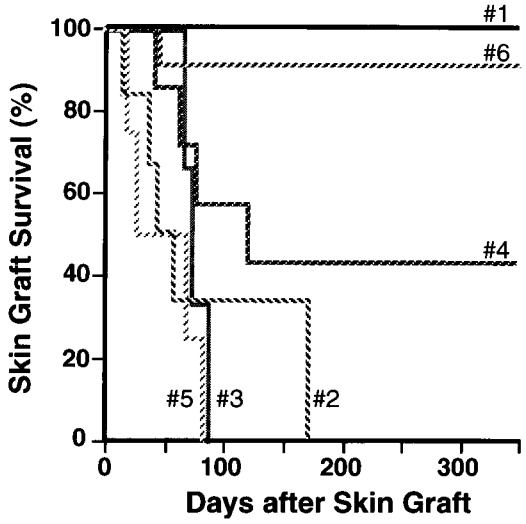
Measurement of Cytokine Production. Supernatants were collected from cultures in which $CD4^+$ cells (1×10^6/1 ml) isolated from the recipients were incubated for 24 or 48 hr with irradiated (15 Gy) spleen cells (2×10^6/1 ml) collected from the donor strain. Sandwich ELISAs of IFNγ, IL-2, and IL-10 were carried out using antibodies and recombinant cytokines purchased from Genzyme.

Analyses of Chimerism. The chimerism was evaluated by using a FACScan (Becton Dickinson) after three color-staining with mAbs. In brief, cells were stained with fluorescein isothiocyanate-labeled mAbs to donor MHC class I (H-2K, Meiji Milk Products, Tokyo, Japan), antigens and phycoerythrin-labeled mAbs to recipient H-2K antigens (PharMingen). Cells nonspecifically labeled with both fluorescein isothiocyanate and phycoerythrin were excluded from counts. The lineage markers were detected as the third color after staining with biotinylated mAbs to the responding antigens (CD3-ε, clone 145–2C11; CD11b, clone M1/70; CD11c, clone N418; CD45R/B220, clone RA3–6B2; PharMingen) and the streptavidin-Cy-chrome (PharMingen).

We have carried out many experiments in several different donor and recipient mouse combinations. For these, we used BALB/c, CDF1, or C3H mice as allogeneic donors or recipients. In all of these experiments, the results obtained were impressively similar to those in the (BALB/c → C57BL/6J) combination. Therefore, the data using the (BALB/c → C57BL/6J) chimeric mice are the only data presented in this paper. The number of mice used in each experiment is shown in the figure legends, except Figs. 2 and 6.

RESULTS

Skin Graft Survival Rates. Because the skin is more immunogenic than most other organs (22) and also easy to assess for graft survival, we carried out skin grafts between mice of the strains (BALB/c → B6). These two strains of mice are incompatible at the entire major plus multi-minor histocompatibility barriers. We first examined whether tolerance could be achieved by p.v. injection of cells and then whether the tolerant mice could regularly be produced and persistently maintained by p.v. injection alone or by p.v. injection of SPLCs or BMCs followed by i.v. injection of cells from these sources. As shown in Fig. 1, tolerance was induced but not maintained by p.v. injection alone when only SPLCs (group 3) or BMCs (group 5) were given by the p.v. route as the tolerance-inducing elements. We previously have found that tolerance detected by the delayed-type hypersensitivity response is significantly prolonged by an additional i.v. administration of BMCs (19). Therefore, we injected donor BMCs (3×10^7) i.v. on day 5 after p.v. injection to provide and recruit donor hematopoietic stem cells (HSCs) as a renewable resource. Day 5 is the day when the responses of host T cells against donor alloantigens are at a minimum in the MLR (18). As shown in groups 1 and 4, the graft survival rate was significantly improved by an additional i.v. administration of BMCs. Recipients injected via the p.v. with SPLCs plus the i.v. with BMCs (group 1) showed a 100% skin graft survival rate that lasted >350 days after transplantation. As shown in Fig. 2, luxurious hair growth was observed and the hair growth was counterpoint to the normal hair growth. In most experiments, skin of color different from that of the normal hair of the recipient strain was used. Furthermore, all recipients in group 1 accepted secondary grafts of the donor BALB/c skin lasting for >100 days when the latter were placed on day 238 after the first skin graft. In group 2, SPLCs instead of BMCs were tried as a second treatment given as an i.v. injection but very long-term graft survival was infrequent. This finding suggested that the injection of HSCs among cells injected i.v. was critical because the bone marrow contains more HSCs than does the spleen. The



Group	P.V. Day0	Immunosuppressants Day2	Immunosuppressants Day5	I.V. Day5
#1 (——)	SPLCs	-	-	BMCs
#2 (~~~~)	SPLCs	-	-	SPLCs
#3 (——)	SPLCs	-	-	-
#4 (——)	BMCs	-	-	BMCs
#5 (·····)	BMCs	-	-	-
#6 (- - - -)	BMCs	CsA	CsA	BMCs

FIG. 1. Skin graft survival rates. B6-recipient mice received the following treatments: P.V., p.v. injection with BALB/c SPLCs (3×10^7) or BMCs (3×10^7) on day 0; CsA, administration (i.p.) with CsA (10 mg/kg) on day 2 and day 5; I.V., i.v. injection with BALB/c BMCs (3×10^7) on day 5. All recipients were grafted with BALB/c skin on day 7. Each group consists of >10 experiments.

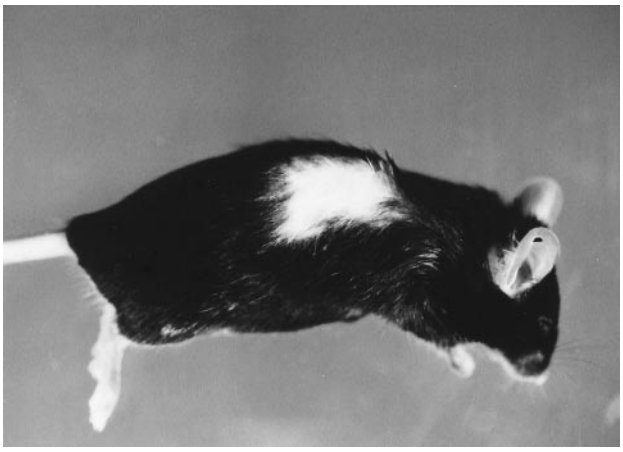
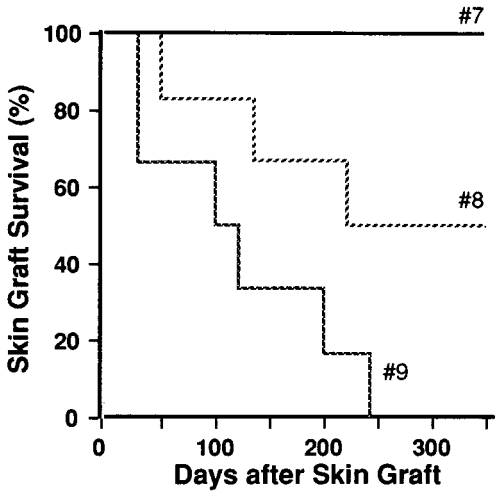


FIG. 2. A representative BALB/c skin graft on B6 recipient: BALB/c skin graft on a recipient 240 days after grafting. The B6 mouse was injected via the p.v. with BALB/c SPLCs on day 0, i.v. injected with BALB/c BMCs on day 5, and grafted with BALB/c skin on day 7.

graft survival rate in recipients injected via the p.v. with BMCs instead of SPLCs also was improved by an additional peripheral i.v. injection of BMCs (group 4) but not as markedly as in mice of group 1—the group that had been injected with SPLCs p.v. plus BMC i.v. 5 days later.

Necessity of Donor T Cells for Skin Graft Acceptance. To elucidate the advantage SPLCs as tolerance facilitators by the first injection, we analyzed the cell population among the SPLCs, which serve to enhance the induction of tolerance. Recipient mice were injected via the p.v. with spleen T cells or with T cell-depleted SPLCs. Recipients that received T cell-enriched SPLCs (Fig. 3, group 7), showed a 100% graft survival rate (>350 days), whereas the graft survival rate decreased when T cell-depleted SPLCs (Fig. 3, group 8) were used. The graft survival rate was significantly reduced when the T cells had been irradiated (15 Gy) before p.v. injection (Fig. 3, group 9). From these results, it can be suggested that the donor T cells play a crucial functional role in enhancement of the skin graft survival. Because BMCs (containing ≈2% T cells) are less active at inducing the tolerant state than SPLCs (containing ≈30% T cells), we postulate that donor-derived T cells are necessary for prevention of rejection of donor-derived hematopoietic cells, including HSCs. Indeed, we also found that peripheral blood lymphocytes injected via the p.v. can be used to induce the tolerant state in place of SPLCs (data not shown).

Influence of Immunosuppressants on Graft Survival. Wojcik and Gorczynski (23) have reported that administration of a single dose of CsA after p.v. injection significantly prolongs the survival of donor skin grafts across minor HC barriers. We therefore examined whether such an immunosuppressant is useful for maintaining the tolerance induction across MHC barriers when BMCs are used. We first administered 10 mg/kg CsA on days 2 and 5 after p.v. injection. As shown in Fig. 1 (group 6), the tolerance induced by p.v. injection of BMCs was significantly enhanced by the administration of CsA. We next examined the effects of another immunosuppressant, FK506, which exerts an influence similar to that exerted by CsA (24). FK506 (1 mg/kg) had an effect similar to that of CsA (data not shown). However, an antimitotic reagent, cyclophosphamide (CY), which has been reported to induce or enhance donor-specific tolerance (25), exerted no action to prolong skin allograft survival in the model under study here (data not shown). These findings indicate that immunosuppressants such as CsA and FK506, even when given only twice, are useful for enhancing the development of the tolerant state. We next examined the critical time to administer these immunosuppressant agents



Group	P.V. Day0	Immunosuppressants Day2	Immunosuppressants Day5	I.V. Day5
#7 (——)	T cell-enriched SPLCs	-	-	BMCs
#8 (- - -)	T cell-depleted SPLCs	-	-	BMCs
#9 (——)	Irradiated T cells	-	-	BMCs

FIG. 3. Effects of donor T cells on skin graft survival rates. B6-recipient mice received the following treatments: P.V., p.v. injection (3×10^7) with BALB/c T cell-enriched SPLCs (group 7), T cell-depleted SPLCs (group 8), or irradiated T cells (group 9) on day 0; and I.V., i.v. injection (3×10^7) with BALB/c BMCs on day 5. All recipients were engrafted with BALB/c skin on day 7. Groups 7 and 8 comprised experiments on six mice each and group 9 experiments on five mice.

after the p.v. injection of BMCs. The tolerant state was enhanced by CsA when it was administered at any time between day 0 and day 5 after the p.v. injection of BMCs, but administration on day 2 appeared to be the most effective time for administration of this agent (data not shown). The administration of CsA on the day before or on day 7 after the p.v. injection did not enhance tolerance induction. In some experiments, BMCs were injected only i.v. (but not p.v.). The graft survival rate in these experiments was very poor, even though CsA was administered (data not shown). This finding strongly suggests that the p.v. injection step is critical to the induction of long lasting allograft tolerance in this model.

Induction of Donor-Specific Clonal Anergy in $CD8^+$ T Cells of Graft-Accepting Recipients. Next, we attempted to examine the mechanisms underlying the tolerance induced by these procedures. As shown in Fig. 4*A*, the proliferative responses of $CD8^+$ cells from the graft-accepting B6 (H-2^b) recipients against the donor BALB/c (H-2^d) alloantigens were significantly lower than those of the graft-rejecting recipients and also those of normal B6 controls, whereas the responses against third party (C3H: H-2^k) cells were vigorous. Hyporesponsiveness of the $CD8^+$ cells against donor alloantigenic cells to which tolerance had been produced, however, was found to be completely normalized if IL-2 (50 units/ml) was added exogenously to the MLR assays (Fig. 4*A Center*). Furthermore, generation of CTLs against donor MHC (H-2^d: P815) target cells also was significantly low when $CD8^+$ cells from the graft-accepting recipients were analyzed (Fig. 4*B*)

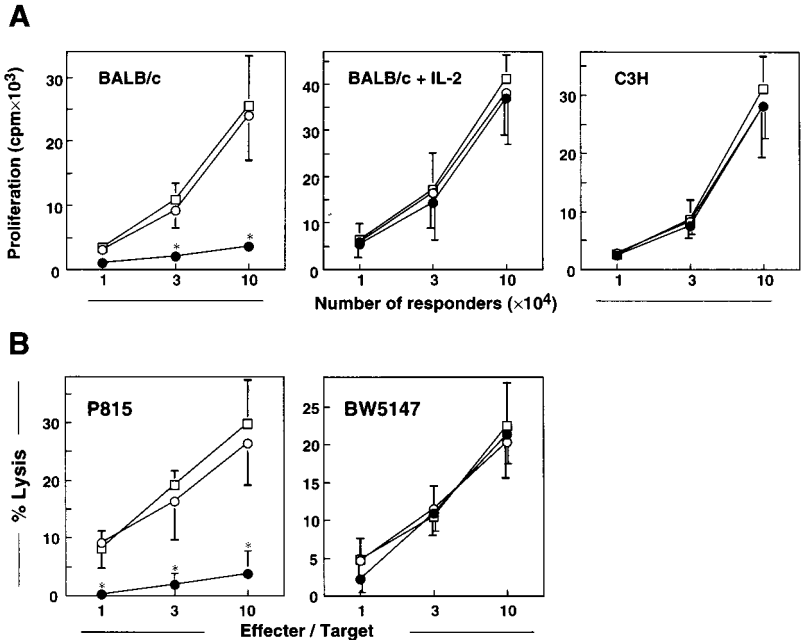


FIG. 4. MLR and generation of CTLs in recipients after skin grafts. Closed circles (●) represent the responses of T cells from the recipients (eight mice) accepting donor skin grafts. Open circles (○) represent responses of T cells from the recipients (seven mice), which rejected the graft. Open squares (□) represent untreated B6 (four mice). *(A)* MLR responses of isolated $CD8^+$ cells against indicated stimulators. *(B)* CTL responses of isolated $CD8^+$ cells against indicated target cells. *, $P < 0.001$ compared with two other populations by the Tukey–Kramer multiple comparisons test.

and compared in this cytotoxic assay to H-2^d target cells to which the host had either been sensitized or had not been tolerized. Responses of $CD4^+$ cells from the graft-accepting recipients were slightly low as compared with those of recipients not accepting the grafts (data not shown). These findings indicate that donor-specific clonal anergy is induced in the $CD8^+$ CTLs of the recipients.

Dominance of $CD4^+$ Th2 Cells in Graft-Accepting Recipients. Gorczynski *et al.* (26) recently have found that the donor-specific tolerance induced after p.v. injection of minor HC-disparate cells is associated with decreased production of the Th1 cytokines (IFNγ and IL-2) and that this enhances the production of the Th2 cytokines (IL-4 and IL-10). Furthermore, after treatment with anti-IL-10 antibody or IL-12, the prolongation of graft survival was found to be eliminated (27). Other researchers analyzing transplantation of vascularized grafts also have reported that recipients exhibiting accommodated grafts show a Th2-type cytokine response, whereas the recipients that have rejected grafts show a Th1-type cytokine response (8, 9). Therefore, we analyzed the cytokine production in the cellular responses against donor alloantigens to determine which type of Th cells are dominant in our tolerance system. $CD4^+$ cells were isolated from tolerant and nontolerant recipients. The $CD4^+$ cells were cultured together with irradiated BALB/c SPLCs, and the production of IL-2, IL-10 and IFNγ was measured in the culture preparations. As shown in Fig. 5, the production of both IL-2 and IFNγ in the cultures of $CD4^+$ cells from graft-accepting recipients was significantly lower than was the production of IL-2 and IFNγ by $CD4^+$ cells of the nontolerant recipients. By contrast, the production of IL-10 was significantly higher in the $CD4^+$ T cells of the tolerant mice as compared with that of $CD4^+$ cells from the nontolerant mice. The dominance of the Th2 cytokine production by the $CD4^+$ cells from the graft-accepting recipients was not seen when C3H SPLCs were used as stimulators (data not shown). Based on these findings, we conclude that $CD4^+$ Th2 cells are the dominant T cells in the graft-accepting recipients.

Microchimerism in the Graft-Accepting Recipients. Starzl *et al.* (28, 29) have observed that, in patients who accept liver

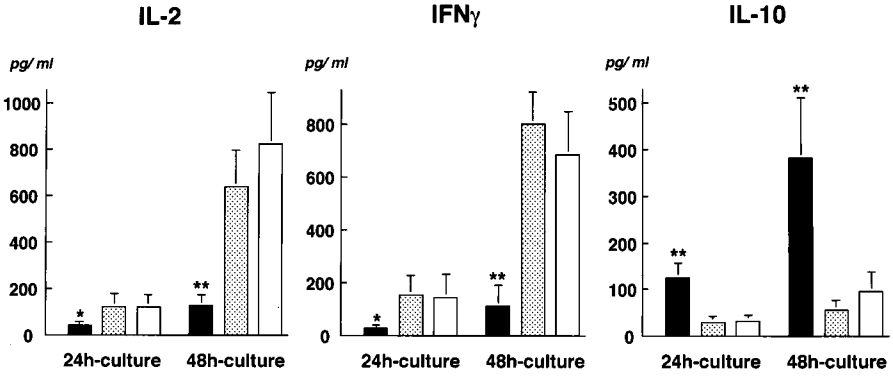


FIG. 5. Cytokine production from isolated $CD4^+$ cells in response to the donor alloantigens. Closed bars represent the production of the indicated cytokine by the $CD4^+$ cells from the recipients (eight mice) accepting donor skin grafts. Dotted bars represent production of the indicated cytokine by the $CD4^+$ cells from the recipients (seven mice), which rejected the graft. Open bars represent B6 mice (four mice) immunized with BALB/c SPLCs. **, $P < 0.001$ and *, $P < 0.05$ compared with two other populations by the Tukey-Kramer multiple comparisons test.

allografts, donor-dendritic cells and macrophages migrate into the host lymph nodes, skin, and heart from the grafted liver. They have concluded that such systemic microchimerism may induce a clonal deletion or suppression of the donor alloantigen-reactive T cells. Because a second i.v. injection of BMCs significantly enhanced the tolerance induced by the p.v. injection in our experiments (as shown in Fig. 1), the cells among the donor BMCs that differentiated from HSCs might be considered to have established a degree of chimerism, probably a microchimerism, in the tolerant recipients, and this michrochimerism may play a crucial role in the maintenance of the tolerant state that has been established. Skin-accepting recipients were therefore analyzed for chimerism using the hepatic mononuclear cells or mononuclear cells of spleen and BM. The skin-rejecting nontolerant recipients also were analyzed for chimerism. Cells ($CD4^+$, $CD8^+$, $B220^+$, and Mac-1^+ cells) with donor MHC phenotype (H-$2k^d$) were readily detected only among the hepatic mononuclear cells of the graft-accepting recipients (Fig. 6). The donor cells also were found in very small numbers in the spleen and BM but not in the recipients that had rejected the grafts (data not shown). These findings indicate that chimerism has been established in the graft-accepting recipients and the donor cells present in the host liver may act in a major way to facilitate the induction of tolerance.

DISCUSSION

In the present study, using p.v. injection of allogeneic cells, we have developed and evaluated protocols which regularly permit the induction of donor-specific, impressively persistent (>350 days) tolerance for skin grafts across major plus multiminor HC barriers. Antigens administered orally or via the p.v. are known under certain conditions to exhibit low or no immunogenic influence (Chase–Sulzberger effect) (30). Applying this model, various attempts have been made to induce donor-specific tolerance across MHC barriers, which will prevent rejection of allografts, such as the heart (11, 12), kidney (14), and pancreas (13). However, although the survival rate of such allografts certainly has been prolonged, almost all of the allografts in these previous studies ultimately have been rejected. Long lasting (>100 days) liver graft survival induced by p.v. injection of donor SPLCs has been reported in rats (15). Kamada (31), however, has reported that orthotopic liver transplantation itself induces donor-specific tolerance, suggesting that p.v. injection may not be necessary or may be accomplished by the transplant itself when liver transplantation is being investigated. We present herein evidence for regular induction of long persisting (<350 days) tolerance for skin allografts across major plus multi-minor HC barriers using p.v. injection of allogeneic cells in mice.

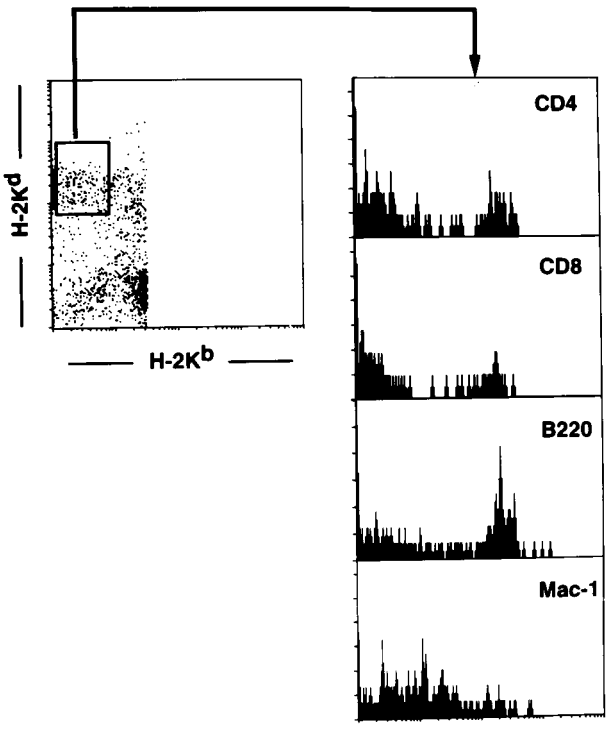


FIG. 6. Staining profiles of hepatic mononuclear cells collected from graft-accepting recipients. Donor cells are detected as donor MHC (H-$2K^d$-phycoerythrin)-positive and recipient MHC- (H-$2K^b$-fluorescein isothiocyanate) negative cells. Lineage markers (such as CD4/8, B220, and Mac-1) are detected by the third color. Cy-chrome labeling with the corresponding mAbs.

In our studies, tolerance across the MHC barrier appears mainly to be associated with clonal anergy caused by the absence of signals from costimulatory molecules in an interaction between host T cells and antigen-presenting cells, as described (18). This tolerance appears to be maintained by suppression of the function of donor-reactive Th1 cells via a dominant influence of their Th2 counterpart. Sayegh *et al.* (9) have reported that donor-specific Th2 but not Th1 cells expand after the induction of anergy by blockage of the CD28-B7 interaction. Gorczynski *et al.* (32) recently have found that a subset of $\gamma\,\delta^+$T cells produces Th2 cytokines and that these regulate mouse skin graft rejection after p.v. preimmunization. We have previously found that, even when injected i.v., BMCs accumulate mainly in the host liver but not in the bone marrow or spleen; many more cells accumulate in the host liver after the i.v. injection of allogeneic BMCs than after an i.v. injection of allogeneic spleen cells or thymocytes (19). Therefore, an additional i.v. injection of donor BMCs has been tested and found to supplement the donor cells present in the host liver in facilitating induction of anergy in the donor-specific CTLs. The donor BMCs also may stimulate the $\gamma\,\delta^+$T cells to produce Th2 cytokines. Indeed, in our previous investigation, the donor-specific delayed-type hypersensitivity mediated by Th1 cells was persistently suppressed by the additional i.v. administration of BMCs in our model. The dominance of the Th2 component may be facilitated by the administration of CsA, a potent inhibitor of IL-2 (33) and IFNγ (34) production, which spares the production of the Th2-type cytokines (35, 36).

In the present study, we regularly performed the skin grafts on day 7 after the p.v. injection of SPLCs. However, entirely equivalent results have been observed in graft survival when the skin grafts are placed on the recipients on the same day as the p.v. injection, and thus permitting the essential features of the protocol to be completed in one day with obvious advantages. Already, in addition to skin allografts, allopancreatic tissues transplanted under the renal capsules of recipient mice have been accepted and associated with production of lasting immunologic tolerance when we used this single-day protocol (H.M., unpublished observations). A single-day protocol may become important in the future because, with the essential features of the method we describe herein, a single-day protocol might prove more applicable to organ transplantation in humans than a protocol that requires several days to complete.

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